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REVIEW

# Modeling multiple sclerosis in laboratory animals

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**Abstract** Inflammatory demyelinating disease of the central nervous system is one of the most frequent causes of neurological disability in young adults. While in situ analysis and in vitro models do shed some light onto the processes of tissue damage and cellular interactions, the development of neuroinflammation and demyelination is a far too complex process to be adequately modeled by simple test tube systems. Thus, animal models using primarily genetically modified mice have been proven to be of paramount importance. In this chapter, we discuss recent advances in modeling brain diseases focusing on murine models and report on new tools to study the pathogenesis of complex diseases such as multiple sclerosis.

**Keywords** Autoimmunity · Immunopathology · EAE · TH17 · Cytokines · TH1 · IL-12 · IL-23 · IL-18 · CD8 · CTL · Gene targeting · Transgenic · *Mus musculus* · Animal models · In vivo · Multiple sclerosis

## Experimental autoimmune encephalomyelitis—the gold standard

### Mode of induction

Experimental autoimmune encephalomyelitis (EAE) was first described and established as an important model of central nervous system (CNS) autoimmune inflammation over 50 years ago [1]. EAE can be actively induced in susceptible inbred mice like SJL mice by immunization with mouse spinal cord homogenate [2], myelin basic protein (MBP; [3]), proteolipid protein (PLP; [4]) or peptides corresponding to the major encephalitogenic regions of MBP ($MBP_{84–104}$), PLP ($PLP_{139–151}$ or $PLP_{178–191}$), or myelin oligodendrocyte glycoprotein ($MOG_{92–106}$) in complete Freund's adjuvant (CFA). MOG makes up less than 0.5% of all myelin proteins and is situated on the surface of myelin sheaths. $MOG_{35–55}$ peptide is strongly encephalitogenic in C57BL/6 mice, the strain providing the genetic background of most transgenic mice. Disease induction with intact MBP or $MBP_{84–104}$ in SJL mice, and likewise with $MOG_{35–55}$ in C57BL/6 mice, requires the use of pertussis toxin (PTX) as part of the induction regimen. PTX has been hypothesized to facilitate immune cell entry into the CNS [5, 6]; however, PTX has other biological effects that could contribute to its activity in EAE, such as breaking T cell tolerance and promoting clonal expansion and cytokine production by T cells [7–9]. In most murine EAE models, after a prodromal interval of 10–20 days, paralysis of tail and hind legs, progressing to the forelimbs, and weight loss develop, reflecting preferential targeting of inflammation to the spinal cord and to some extent the cerebellum (referred to as "classic EAE"). Accordingly, the scoring system is based primarily on motor deficits resulting from spinal cord lesions. However, enhanced brain inflammation and atypical clinical variations have been described in murine models using certain strain/antigen combinations or a background of genetic interferon (IFN)-$\gamma$ deficiency [10, 11]. Factors that can influence the manifestation of disease are age, gender, and season at immunization [12, 13], the preparation and physical

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structure of antigen/adjuvant emulsions [14, 15], the species origin of the antigen [16], and epigenetic factors [17]. The disease course of EAE depends on the strain, immunogen, and use of PTX [18]. In the SJL (H-$2^s$) mouse strain, EAE displays a relapsing–remitting clinical course (RR-EAE), while in C57BL/6 (H-$2^b$) or PL/J and B10.PL (H-$2^u$) mouse strains, the disease is chronic-progressive (C-EAE) or acute monophasic, respectively. Alternatively to direct induction, EAE can also adoptively be transferred to naïve mice by injection of in vitro neuroantigen-activated T cells isolated from primed donors [19]. The most common adoptive transfer models involve major histocompatibility complex (MHC)-class-II-restricted CD4+ T helper ($T_H$) cells [20], while only a few reports describe EAE transfer by CD8+ T cells [21, 22]. Many of the pathogenic CD4+ $T_H$ cells respond to activation by secreting IFN-$\gamma$ and tumor necrosis factor (TNF)-$\alpha$, but not interleukin (IL)-4 and IL-5, qualifying them as $T_H1$ cells. Recent studies, however, suggest that this population includes a subset of CD4+ $T_H$ cells that preferentially produces IL-17 ($T_H17$ cells); by modifying T cell effector functions in vitro before transfer, Langrish and colleagues confirmed that IL-23-driven IL-$17^+$ T cells are highly encephalitogenic [23]. It therefore currently appears that both $T_H1$ and $T_H17$ cells have pathogenic potential [24]. Another way of manipulating the myelin-specific T cells in vitro before transfer is the labeling by genetic markers or fluorescent proteins/dyes, so that the autoreactive T cells can be traced in the recipient [25, 26].

No spontaneous and naturally occurring animal models of spontaneous EAE exist. However, T cell receptor (TCR) transgenic mice have been generated that do not require peptide priming and strong immune adjuvants but spontaneously develop EAE with variable incidence. Although the immunological repertoire in TCR transgenic mice is significantly altered and biased towards enhanced antigen-specific responses and alternative mechanisms of immune regulation [27, 28], studies using such transgenic models have contributed significantly to the understanding of the pathogenesis of CNS autoimmunity. Various TCR transgenic models of EAE on different genetic backgrounds exist, including humanized mice carrying human leukocyte antigen (HLA) class II molecules associated with multiple sclerosis (MS) susceptibility and TCR [29–31]. Interestingly, HLA-DRB1*0401-restricted $MBP_{111–129}$-specific humanized TCR transgenic mice display clinical signs of inflammatory demyelination in brain stem and cranial nerve roots, like dysphagia in addition to ascending paralysis typical of EAE [31]. 19G B10.PL (H-$2^u$)/$MBP_{Ac1-11}$, [27], 5B6 SJL (H-$2^s$)/$PLP_{139–151}$ [32], and 2D2 C57BL/6 (H2-$^b$)/$MOG_{35–55}$ TCR transgenics [33] are spontaneous models utilizing mouse MHC class II restriction elements. The clinical spectrum displayed varies from classical hind limb paralysis to isolated optic neuritis (2D2 TCR transgenics in the C57BL/6 strain). A large percentage of $MOG_{35–55}$ TCR transgenic mice crossed to MOG-specific immunoglobulin heavy chain knock-in animals spontaneously develop a Devic-like, opticospinal disease [34, 35].

## Pathogenesis (priming and CNS invasion)

Classical models of EAE are mainly driven by MHC-class-II-restricted CD4+ T lymphocytes [36–38]. Active and passive induction protocols both activate peripheral myelin-specific CD4+ T cells that have escaped immune tolerance and circulate in the periphery of naïve animals (reviewed in [39]). During the induction phase of actively induced EAE, myelin-reactive CD4+ T cells are primed and expand in the peripheral lymphoid organs. In contrast, in the adoptive transfer model of EAE, disease is induced by the peripheral introduction of a preactivated population of myelin-epitope-specific CD4+ T cells to a naïve mouse. The effector phase involves migration of activated myelin-specific T cells to the CNS, where they cross the blood–brain barrier (BBB). CNS-infiltrating CD4+ T cells require myelin peptides presented by local antigen-presenting cells (APCs) for full reactivation [40, 41]. Recently, also dendritic cells (DCs) have been demonstrated to be the initial APCs for encephalitogenic T cells to recognize their target antigen (Ag)/organ [42, 43]. The reactivated CD4+ T cells then initiate a cascade of events, including the secretion of chemokines, that recruit predominantly macrophages to the site of inflammation. Proinflammatory cytokines secreted by macrophages, such as TNF-$\alpha$ and IL-1, are important for both perpetuating inflammation and contributing to CNS tissue damage [44]. Microglia are also activated in EAE and blocking microglial activation suppresses the development of EAE, supporting their pathogenic role in CNS autoimmune disease [45–48]. The function of astrocytes in disease pathogenesis remains unclear, as they show proinflammatory activity by producing neurotoxic mediators, cytokines, and chemokines but also exert neuroprotective functions by providing neurotrophic factors (reviewed in [49–51]). After CNS tissue is damaged, epitope spreading is believed to occur by the release and subsequent presentation of endogenous myelin epitopes by local APCs [26, 52] and has been shown to mediate clinical relapses in certain EAE models [53, 54].

## Mouse, rat, monkey background

EAE is mainly studied in the highly reproducible murine model, as mice are inbred and a genetically homogenous population. Furthermore, transgenic and targeted gene deletion murine models are abundant,

and numerous reagents are available that can be used to dissect the pathogenic mechanisms in EAE. Nevertheless, EAE has been replicated in a wide range of species, and the different species and strains offer different advantages. EAE induced in rabbits or guinea pigs with CNS myelin is similar to MS in that inflammation occurs in both the brain and spinal cord [55, 56]. To investigate the contribution of demyelinating antibodies, EAE models in guinea pig [57], common marmoset [58], and certain rat and mouse models [16, 59] have been proven to be useful.

EAE is induced in the marmoset by immunization with CNS tissue homogenate or recombinant MOG in CFA (reviewed in [60]) and provides a disease model that reproduces several clinical and pathological features of MS. Adoptive and passive transfer experiments indicate that, similar to MOG-induced EAE in the rat, synergism between encephalitogenic T cells and demyelinating antibodies is required for full lesion formation [58, 61]. In spite of variable disease induction in the outbred marmoset species and limited availability of reagents, the marmoset model may have some value for drug testing considering that it is phylogenetically closer to humans than rodents.

### Histopathological findings—man versus mouse

Histopathological changes seen in EAE appear to represent a stereotypic response of the CNS to autoimmune damage. While EAE, as many other disease models, certainly is not a full mirror image of MS, it comes pretty close to what can be seen in human MS lesions. However, the clinical features, including the pattern of disease progression and the histopathology, may vary depending on (1) the species in which disease is provoked as well as (2) the approach of EAE induction (for review, see [62]).

Early and/or acute MS lesions in humans are characterized by demyelination (Fig. 1a, b), extensive macrophage invasion (Fig. 1c, d), perivascular and parenchymal T cell infiltrates (Fig. 1g, h), and few B cells/plasma cells as well as relative axonal preservation. While ongoing remyelination next to demyelination is a typical feature of active MS lesions [63], based on the myelin loss, extent of oligodendrocyte preservation, and composition of the inflammatory infiltrates, four distinct—admittedly descriptive—histopathological patterns of acute MS lesions have been suggested [64] and await final clinical validation. In brief, common feature of these acute lesions is the predominance of macrophages, which are ~10 times more frequent than, e.g., T cells (the latter are mainly of the CD8+ phenotype). On top of which, some lesions (referred to as pattern II) display activated complement on dying myelin sheaths and in macrophages, while others (so-called pattern III) are described to have a preferential loss of the myelin-associated glycoprotein (for review, see [65]). Recent data also point to a crucial role of DCs, which due to their close proximity to invading T cells adjacent to CNS vessels (Fig. 1f, g, h) seem to be crucial APCs in conferring CNS inflammation of encephalitogenic T cells [42]. In contrast to acute MS lesions, chronic MS plaques display extensive and sharply demarcated white matter lesions with variable axonal loss and significant fibrillary astrogliosis; due to only few residual lymphocytes and macrophages/microglia, these chronic lesions appear to be "burnt out" (for review, see [65]).

Acute EAE lesions in mice—the species which we primarily refer to—histopathologically resemble the perivascular macrophage-/microglia-rich inflammatory pattern of acute/chronic active MS lesions in humans (Fig. 2), while severe demyelination is seen rather in chronic relapsing EAE models (for review, see [62]).

Due to the high reproducibility of EAE lesions with respect to cellular composition and histopathological appearance, animal models of EAE are most valuable in studying pathogenetically relevant consequences of defined manipulations. Moreover, such lesions can be studied at any time point, which explains why EAE models also in terms of histopathology are a most precious addition to the repertoire of modern MS research tools.

## Use of transgenic mice to unravel pressing questions about the pathogenesis of CNS inflammation; knockout mice

Specific mouse genes can routinely be overexpressed as transgenes or eliminated by gene targeting ("knockouts"), which allows a "clean" and direct assessment of the causative roles of certain molecules in disease pathogenesis. Various transgene constructs involving targeted expression of MHC/costimulatory molecules or alterations of the TCR repertoire towards a highly autoreactive population have been designed to challenge the immune system's mechanisms of self-tolerance [66]. Studies in transgenic and knockout mice have implicated a number of effector molecules such as cytokines, chemokines, and other proteins in MS [67]. The conventional approach has several disadvantages; transgene expression may have direct toxic effects on the target tissue and be insensitive to regulatory networks, and in particular, normal developmental kinetics cannot be mimicked, as expression is either "on" or "off" from time of developmental onset (reviewed in [68–71]). Further (conditional) techniques have been developed to limit manipulated gene expression to defined cell types (Cre-loxP system of site-specific recombination [72]) and specific time points. Commonly used examples for such a spatial and temporal control of genes of interest are the

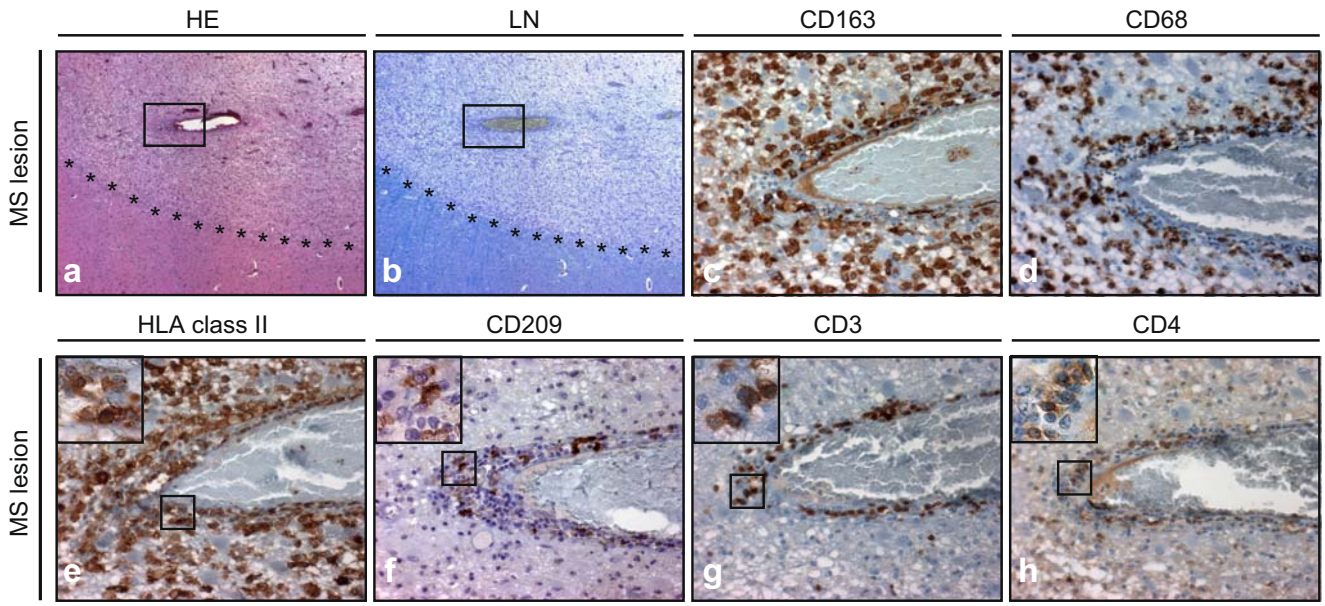


**Fig. 1** Histopathology of acute and/or chronic active MS lesion: severe demyelination (H&E (**a**); *LN*, Luxol-Nissl (**b**)) and numerous CD163+ (**c**) or CD68+ (**d**) macrophage and microglia-rich inflammatory infiltrates including prominent perivascular inflammation are predominant histopathological findings. Gray and white matter in **a** and **b** is separated by *asterisks*. *Rectangles* represent the area chosen for higher-magnification analysis throughout. While strong HLA class II immunoreactivity (**e**) was present throughout the MS lesion, CD209+ cells (**f**) were mainly restricted to vessels and seen in close proximity to invading CD3+ (**g**) and CD4+ (**h**) T cells. *Scale bar* 400 μm (**a**, **b**), 100 μm for all other panels. Adapted from [42]

tetracycline-controlled transactivator system [73] and the tamoxifen-controlled expression of Cre (CreERT2, [74]). CNS-specific expression can be induced, e.g., by promoter regions encoding glial fibrillary acidic protein (GFAP), neurofilament, MBP, or MOG which are expressed in astrocytes, neurons, and oligodendrocytes, respectively. The nestin-Cre transgene mediates excision of loxP-flanked sequences in early neuronal precursors during embryonic life, resulting in target gene inactivation in all neuroectodermal cells of the CNS [75]. CNS-targeted manipulations of cytokine/chemokine expression offer a relatively nonintrusive technique for assessment of their role (reviewed in [68, 76]). In vivo cell lineage ablation, e.g., of $MOG^{+}$ oligodendrocytes, has recently been improved by stable mouse lines, carrying conditional expression constructs for diphtheria toxin (DT) or DT receptor (DTR) that could be activated upon Cre-mediated recombination and the application of DT, respectively [77]. CD11b-herpes simplex virus thymidine kinase (HSVTK) transgenic mice, which express HSVTK in macrophages/microglia

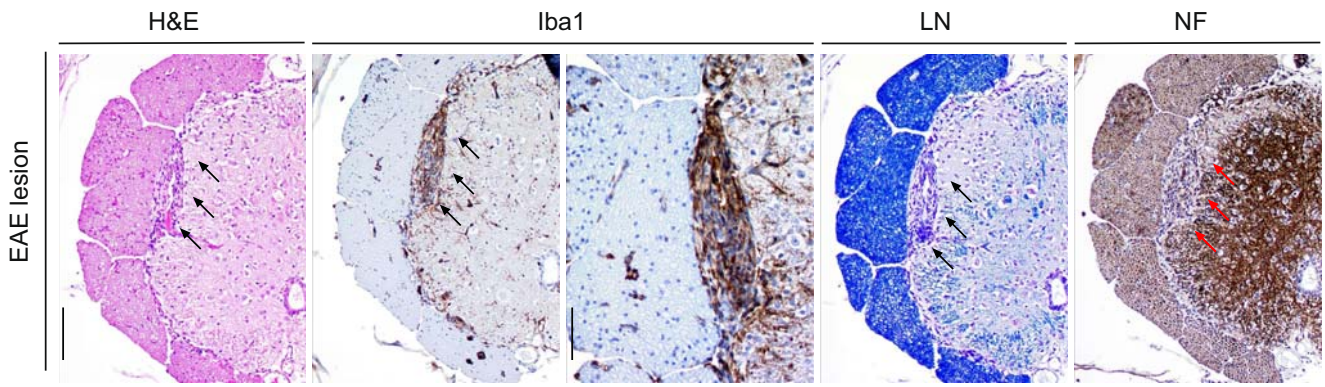


**Fig. 2** Spinal cord cross sections of wild-type mice displaying an acute perivascular EAE lesion (H&E, *arrows*), mainly consisting of activated Iba1+ macrophages/microglia (*second* and, at higher magnification, *third row*). Impairment of myelinated (Luxol-Nissl, *LN*; *fourth row*) and axonal structures (neurofilament, *NF*, *fifth row*), while indicated due to the early state is not yet fully developed (and better visible in chronic lesions). *Scale bars* 100 μm for *first*, *third*, *fourth*, and *fifth column*; 100 μm for *second*, *fourth*, and *sixth column*. Adapted from [96]

have been generated as a tool to study the impact of microglial activation on CNS disease in vivo. Expression of HSVTK renders preferentially proliferating cells sensitive to ganciclovir, as the active metabolite competes with thymine for DNA synthesis [45]. To dissect APC functions in the CNS parenchyma versus the periphery, gene targeting can also be combined with bone marrow chimerism [42]. Astrocyte activation during EAE in vivo was monitored by Luo and colleagues by the use of bioluminescence imaging in mice expressing luciferase under the control of a GFAP promoter [78]. "Knock-ins" of functional cytokine genes have been ligated to green fluorescent protein (GFP) for cell tracking of cytokine-producing cells [79]. Based on the linked expression of GFP to $Foxp3^+$ T cells, two groups have generated Foxp3gfp.KI mice in order to reliably monitor regulatory T cells (T-reg) in vivo [80, 81]. Taken together, the true strength of the mouse model is its capacity to intervene with the pathogenesis of inflammatory CNS disease at the molecular level and to prove or mostly dismiss hypothesis about the involvement of specific molecules in the inflammatory cascade.

## Cytokines and mediators of inflammation

Upon cognate antigen recognition on an activated APC, T cells become polarized towards an effector type depending on the quality of priming immune synapse and the cytokines present. Signaling of certain signature cytokines results in lineage commitment towards one of the three effector types, termed $T_H1$, $T_H2$, or $T_H17$. The classic paradigm, proposed by Mosmann and Coffman in 1986, strictly divided effector $T_H$ cells into $T_H1$ cells, directing cell-mediated immunity, and $T_H2$ cells, regulating B cell activity and humoral immunity [82]. $T_H1$ cells produce primarily IFN-γ and IL-2, while IL-4 and IL-5 are produced by $T_H2$ cell. Lineage commitment employs autocrine loops for "self-propagation" and reciprocal inhibition via their cytokines. In addition to the $T_H1$-promoting effects of IFN-γ itself, $T_H1$ cells are predominantly polarized by the APC-derived factors IL-12 and IL-18 [83]. The $T_H1/T_H2$ paradigm provided a conceptional framework which shaped our understanding of communication pathways during adaptive immune response. Over the years, $T_H1$ cells were found to be the main culprits behind CNS inflammation, primarily based on the fact that inflammatory CNS lesions and invading T cells produced IFN-γ. In MS patients, elevated levels of IL-12p40 mRNA were detected in the CNS [84], while in the animal model EAE such $T_H1$ cells were shown to actually infiltrate the CNS in great numbers and produce their effector cytokines when CNS inflammation occurred. Purified $T_H1$ cells could be transferred into naive animals and mediate autoimmune disease. Conversely, $T_H2$ cells were shown to exert beneficial effects in EAE (reviewed in [85]).

Given the overwhelming case for $T_H1$ cells and IFN-γ, it was surprising at first that in mice none of the $T_H1$ and $T_H1$-inducing cytokines (IFN-γ, IL-12, IL-18, TNF-α) could account for the proposed importance of the $T_H1$ effector cells in the disease [86–89]. Even though functional studies could hardly verify the pivotal role of $T_H1$ cells in autoimmunity, the model was not abandoned but clearly dominated common belief for decades. A tangible alternative was proposed with the discovery that the IL-12 family member IL-23 instead of IL-12 itself is a prerequisite for the development of EAE [87, 90, 91]. The central role of IL-23 in EAE was proven by Sedgewick and colleagues by the deletion of the p19 subunit of IL-23 [91]. The particular impact connected to this finding was the description of a novel $T_H$ cell effector type, which showed IL-23-dependent expansion and expression of the proinflammatory cytokine IL-17A [23]. They were therefore coined $T_H17$ cells. IL-23- and IL-6-deficient mice showed impaired $T_H17$ polarization. In consequence, it seems that such loss rendered the mice resistant to EAE [24]. A plethora of data was generated that correlated $T_H17$ cells and IL-17A expression with the development of autoimmune inflammation in mice and humans (reviewed in [92]). Almost forgotten was the fact that in most respects $T_H1$ cells and $T_H1$ cytokines correlate just as well with autoimmune disease and that adoptive transfer of $T_H1$ cells for the passive induction of EAE was the standard procedure for years.

Surprisingly, IL-17A neutralization in EAE in C57BL/6 mice by Hofstetter et al., however, only revealed a very mild effect of treatment with either monoclonal antibodies against IL-17A or with the soluble receptor of IL-17A and F [93]. IL-17A−/− mice were generated by the group of Iwakura and found to be fully susceptible to EAE after active immunization but demonstrate an alleviated course of clinical EAE in the chronic phase of disease [94]. Even more surprisingly, these findings were then interpreted by others to represent the clear and solid proof that IL-17A is the key player in CNS autoimmune inflammation in mice and probably in men as well. The slight change in the course of EAE found upon deletion of IL-17A certainly confirms its proinflammatory properties at the site of an active autoimmune lesion but, nonetheless, fails to mark it as an essential encephalitogenic factor. Haak et al. and Kreymborg et al. have by now dismissed the $T_H17$ cytokines IL-17A, IL-17F, and IL-22 as critical players in the EAE pathogenesis [95, 96]. After an initial confusion in the field, it remains a solid fact that IL-23 is vital for EAE development while $T_H17$ cells and their cytokines display a great deal of redundancy. Recently, McGeachy et al. and Gyülvészi et al. reported that IL-23 promotes the CNS tropism of encephalitogenic T cells [97, 98]. The mechanistic underpinnings of this phenomenon remain to be established.

Hence, while we have clear evidence that a certain quality of T cell effector function permits encephalitogenic T cells access into the CNS, the precise molecular signature remains to be established.

### BBB biology and leukocyte trafficking to the CNS

Under physiological conditions, a limited number of leukocytes enter the CNS (referred to as immune surveillance; [99]). In the course of EAE, BBB dysfunction is associated with increased leukocyte extravasation across CNS postcapillary venules and subsequent leukocyte accumulation in the perivascular space forming inflammatory cuffs. In postcapillary venules, the endothelial cell monolayer and underlying basement membrane, ensheathing astrocyte end feet, leptomeningeal cells, and their associated parenchymal basal membrane all contribute to the BBB [100]. Therefore, EAE presents an excellent model to study leukocyte migration across the BBB into the CNS during autoimmune inflammation. Several techniques have been developed to assess the disruption of the BBB in situ, like immunohistochemical analysis for tight junction proteins, serum-derived proteins (fluorescein isothiocyanate albumin leakage assay, [101]), or Evans Blue diffusion [102, 103]. In vivo tracing studies using genetically or fluorescently labeled leukocytes have been combined with intravital microscopic analysis to study interactions with superficial brain microvasculature [104]. Finally, to further elucidate molecular mechanisms of CNS trafficking neutralizing antibodies, small molecule inhibitors or (endothelial specific) transgenic/knockout models of trafficking determinants like adhesion molecules can be used [105].

In EAE, intravital fluorescence videomicroscopy has revealed an integrin $\alpha_4$- and P-selectin-dependent rolling of leukocytes along CNS vessels, without the involvement of E-selectin as in other tissues [106] and an integrin $\alpha_4\beta_1$ (very late antigen-4, VLA-4)-mediated, G-protein-independent capture of T cells at the endothelial cell surface [104, 107]. The integrin $\alpha_4\beta_1$ ligand, vascular cell adhesion molecule (VCAM-1), as well as intercellular adhesion molecule 1 (ICAM-1), and activated leukocyte cell adhesion molecule (ALCAM) are well-described adhesion molecules expressed on inflamed BBB endothelial cells and seem to be important for leukocyte transmigration [108, 109]. The realization that integrin $\alpha_4\beta_1$/VCAM-1 interactions play a crucial role in leukocyte adhesion to the BBB has led to the development of natalizumab, a humanized anti-$\alpha_4$-integrin antibody, as an effective novel drug for the treatment of MS [110, 111]. More recent data indicate that $\alpha_6\beta_1$ integrin, the major receptor of laminin 4, mediates T lymphocyte migration across the endothelial basement membrane. Elimination of laminin 4 or $\alpha_6\beta_1$ integrin resulted in reduced T cell infiltration and EAE severity [112]. Functional expression of CCL19 and CCL21 occurs in CNS venules surrounded by inflammatory cuffs, and these lymphoid chemokines have been implicated in T lymphocyte migration across the endothelial cell monolayer [113]. Previous studies have suggested that subsequent migration out of the perivascular cuff and into the CNS parenchyma requires the classical inflammatory chemokines, including CCL2 [114–116]. Further, leukocytes use focal matrix metalloproteinase (MMP-2 and MMP-9) activity to penetrate the parenchymal basement membrane [117].

## Models of CD8-mediated EAE

Attention has long been focused on CD4+ T cells because susceptibility of MS is associated with MHC class II genes [118], and CD4 T cells are critical to the induction in most EAE models [119]. However, CD8+ T cells have also been implicated in MS by their higher number and invasiveness into the parenchyma compared to mainly perivascularly clustered CD4+ T cells in MS lesions [65, 120–122], their clonal expansion based on TCR analysis [123, 124], and their pathogenicity in certain EAE models [22, 125, 126], whereas in other models, CD8+ T cell may play a regulatory role and protect against the disease development [123, 127–129]. Data showing that CD8+ T cells can, in some conditions, directly attach to and transect axons support the idea that CD8+ T cells mediate damage [130].

### CD4 bias after immunization

Due to the method of induction of classical EAE with CNS-derived antigen emulsified in adjuvants which favors a CD4+ T cell bias in the population of responding myelin-specific T cells, EAE is not an ideal model to investigate the contribution of CD8+ T cells to disease. The most important component in the adjuvant CFA is heat-inactivated *Mycobacterium tuberculosis*, which induces a prominent MHC-class-II-restricted CD4+ $T_H1$ response [131, 132].

Myelin-specific CD8+ T cells can adoptively transfer EAE, thereby establishing a pathogenic role for CD8+ T cells in EAE and providing a model for analysis of CD8+ T cells in MS. In a study by Huseby et al., adoptive transfer of MBP-specific CD8+ T cells isolated from wild-type C3H mice mediated severe CNS autoimmunity [21]. The symptoms resembled many features of MS not seen in CD4+ T-cell-mediated EAE models, such as ataxia, spasticity, hyperreflexiveness, and loss of coordinated movements. CNS demyelinating lesions predominated in the brain versus the spinal cord, which was quite distinct

from most CD4+ T-cell-mediated models of EAE in which the lesions are mainly found in the spinal cord. Perivascular cuffs were composed of lymphocytes, macrophages, and a few neutrophils. CD8+ T-cell-mediated CNS autoimmunity was independent of CD4+ T cells and largely inhibited by neutralizing IFN-γ activity. Two other groups successfully induced CD8+ T-cell-mediated EAE in C57BL/6 mice by transferring CD8+ T cells specific for $MOG_{37–46}$ into syngeneic mice [22, 126].

### Transgenic models (auto versus neo Ag)

Several transgenic mouse models have been developed to study the pathogenic role of CD8+ T cells in autoimmune CNS inflammation. Brisebois and colleagues have recently shown that endogenous CD8+ T cells were necessary for the development of full-blown neuroinflammation in mice overexpressing the costimulatory ligand CD86 on microglia cells [133]. The majority of T cells in the CNS of mouse mutants that overexpress PLP in oligodendrocytes expressed CD8 [134].

Other systems have employed TCR transgenic T cells recognizing natural or model brain autoantigens in an MHC-class-I-restricted manner. In GFAP–hemagglutinin (HA) × CL4-TCR double transgenic mice, in which the viral (neoself) antigen influenza HA is specifically expressed in astrocytes and enteric glia and in which most CD8+ T cells are HA specific, early death due to an attack on enteric glial cells precluded the analysis of EAE development [135]. Adoptive transfer of HA-specific CD8+ T cells resulted in immune cell infiltration in the brains of transgenic mice, expressing HA in astrocytes, but failed to induce clinical CNS disease [136]. In H-2K-restricted $MBP_{79–87}$ TCR transgenic mice, MBP-specific CD8+ T cells sufficed to induce CNS autoimmunity, but immune tolerance prevented these autoreactive cells from causing disease [137]. Transgenic mice expressing a Borna disease virus (BDV) antigen in either neurons or astrocytes were also tolerant to the neoself antigen. In this model, however, adoptive transfer of activated BDV-specific CD8+ T cells was sufficient to induce disease, which was attributed to the high viral antigen concentrations in the CNS [138].

Mice in which the model antigen influenza HA is selectively expressed in oligodendrocytes have been generated (DKI) to test whether autoreactive CD8+ T cells can contribute to the loss of oligodendrocytes as observed in MS plaques. Crossing these mice to CL4-TCR transgenic mice did not result in spontaneous neuroinflammation, and CL4-TCR transgenic CD8+ T cells rather seemed to remain "indifferent" to the neoself antigen sequestered in the CNS. However, transfer of preactivated HA-specific CD8+ T cells in DKI mice led to inflammatory lesions in the optic nerve, spinal cord, and brain [139]. In another study, intracerebral injection of the influenza virus $HA_{512–520}$ peptide in soluble form in CL4-TCR transgenic mice initiated CNS infiltration by the circulating HA-specific CD8+ T cells. It was suggested that peptide-loaded MHC class I molecules expressed on the luminal side of endothelial cells at the BBB might have triggered transmigration [140]. A similar model for CNS-specific autoimmunity employed ovalbumin (OVA) sequestered as a neoself antigen in the cytosol of oligodendrocytes. Introduction of the MHC-class-I-restricted, OVA-specific OT-I TCR as a second transgene led to spontaneous fulminant demyelinating EAE with MS-like lesions, affecting cerebellum, brainstem, optic nerve, and spinal cord. Endogenously generated OT-1 TCR transgenic CD8+ T cells were highly encephalitogenic, which seemed to depend on a BBB, permeable for naïve CD8 T cells during the first 10 days of life and on the production of IFN-γ. In contrast, OVA-specific CD4+ T cells (OT-II), remained fully ignorant of the self-antigen unless OT-I TCR Tg CD8+ T cells first released OVA from oligodendrocytes for peripheral presentation [141]. Recently, a role for HLA-A-restricted CD8+ T cells in the pathogenesis of an MS-like disease was also demonstrated in humanized mouse models [142, 143].

### Infection-induced CD8 expansion

CD8+ T cells are key mediators in the immune response to many viral infections, and a role for CD8+ T cells in demyelination has been illustrated in viral models of CNS inflammation. CD8+ T cells are essential for clearance of virus but might also become pathogenic and damage CNS tissue. Several mechanisms have been described to explain how viruses might trigger autoimmune disease including adjuvant effects and the provision of viral antigens that are similar to self-antigens and stimulate cross-reactive immune responses (molecular mimicry; reviewed in [144]). Models of molecular identity, although artificial, showed that an immune response to a viral infection could recognize identical antigens presented as self in the CNS, resulting in overt CNS autoimmune disease [145, 146]. During lymphocytic choriomeningitis virus (LCMV) infection, LCMV-specific CD8+ T cells expand greatly [147, 148]. Evans and colleagues generated transgenic mice that expressed the nucleoprotein or glycoprotein of LCMV as self in oligodendrocytes. After peripheral (but not CNS) infection with LCMV, the virus was efficiently cleared, but a chronic CNS autoimmune disease developed characterized by T cell inflammatory lesions with mainly CD8+ T cells, areas of focal myelin loss, marked upregulation of MHC class I and II molecules, and clinical motor dysfunction. Autoimmune responses in the CNS developed

despite the fact that LCMV was not detectable in the CNS, demonstrating that autoimmunity can occur at a site distal to the initiating infection [145]. In other models of molecular identity/mimicry, autoimmune disease is induced by infection with a nonpathological Theiler's murine encephalomyelitis virus (TMEV) variant that was engineered to express the immunodominant self-epitope from myelin PLP peptide ($PLP_{139–151}$) [146] or viral peptides that mimic $PLP_{139–151}$ [149]. Other studies focusing on viral infections of the CNS have revealed that T cells under certain circumstances are able to bypass recognition of peptide–MHC complexes and mediate tissue destruction without TCR engagement [150, 151]. Haring and colleagues used transgenic TCR/RAG2$^{-/-}$ mice with only T cells that are not specific for mouse hepatitis virus to show that activated CD8+ T cells are able to cause bystander tissue damage even in the absence of cognate antigen in the CNS [150].

## Theiler virus and models of demyelination

### TMEV-induced demyelinating disease

TMEV, murine hepatitis virus, and Semliki Forest virus are useful models for understanding the potential viral etiology of MS (reviewed in [152]). Intracerebral infection of susceptible strains of mice, such as SJL, with natural TMEV leads to either rapidly fatal encephalomyelitis (high-neurovirulence strains) or persistent CNS infection and immune-mediated demyelination (low-neurovirulence strains, BeAN, or DA; [153, 154]). Gait disturbances, spastic hind limb paralysis, and urinary incontinency start 30–40 days after infection. Intracerebral injection of virus leads to persistent CNS infection. The level of infectious virus is low during the chronic phase, but abundant amounts of viral RNA and antigen can be detected throughout the lifetime of the mouse [155–157]. During the acute phase of the disease, virus replication is mainly in neurons, whereas during the chronic phase, TMEV persists predominantly in macrophages and glia [155, 158–160]. Virus particles have been identified in oligodendrocytes which have a "dying back" pathology [161]. TMEV virus is known to cause demyelination in infected nude mice that cannot generate mature T lymphocytes [162], also pointing to a direct viral effect on myelin damage.

Upon TMEV infection, a variety of chemokines and cytokines are induced in primary astrocytes via the nuclear factor κB pathway including TNF-α, IL-1, IL-6, CCL2, and CCL5 [163–165]. Inflammatory responses to TMEV infection seem to depend on toll-like receptor (TLR)3 and TLR2 [166, 167], as well as protein kinase R, signaling [168]. The immune response is initially directed against persistent viral antigens, but the chronic phase of the disease is dependent on de novo activation of autoimmune CD4+ T cell responses against CNS myelin that develop locally as a result of the release of myelin antigens, i.e., epitope spreading [26, 169–171]. In SJL mice, reactivity appears to multiple myelin peptides starting with the immunodominant epitope and spreading later to other subdominant myelin determinants in a hierarchical manner [170, 172]. Resistance to persistent CNS infection by TMEV is controlled by multiple genetic loci, with the strongest linkage to the class I H-2D MHC region [173, 174], which indicates a role for CD8+ T cells. Most evidence points towards a protective function of CD8+ T cells via the clearance of TMEV from the CNS [175–178]; however, there is also evidence that MHC-class-I-restricted CD8+ T might be directly cytotoxic to axons and mediate injury [179, 180].

### Cuprizone and lysolecithin, demyelination without autoimmunity

Several toxin-based models of demyelination, including cuprizone and lysolecithin, while not attempting to accurately mimic the pathogenesis of autoimmune CNS inflammation, have proven very useful to study the mechanisms of demyelination and remyelination. In contrast to the systemically administered cuprizone, the membrane-solubilizing, glia-toxic lysolecithin has been used to create focal areas of demyelination by direct injection into defined CNS white matter tracts, which allows for a better control of lesion size and location (reviewed in [181]).

Feeding of cuprizone (bis(cyclohexanone)-oxaldihydrazone) to young adult mice induces a consistent, synchronous, and anatomically reproducible demyelination. Furthermore, removal of cuprizone of the diet of mice leads to remyelination (reviewed in [182]). Cuprizone is a chelator that binds copper, an essential component of metalloenzymes, like the mitochondrial cytochrome oxidase and monoamine oxidase. It has been assumed although not proven that copper deficiency results in specific damage to oligodendrocytes in the CNS and subsequent demyelination. The specific susceptibility of oligodendrocytes has been attributed to the high metabolic demand of these glial cells required to maintain a vast expanse of myelin and the resulting vulnerability to a disturbed energy metabolism.

Genetic factors and gender influence susceptibility to cuprizone-induced demyelination [183]. Cuprizone can induce demyelination in different strains of mice, among them in 8–10-week-old C57BL/6 mice, the background most commonly used for knockout and transgenic mice. A 0.2% dose of cuprizone is usually well tolerated. If

cuprizone is overdosed, (mitochondrial) hepatopathy and weight loss are serious toxic effects [184]. The regional pattern of demyelination includes white matter tracts preferentially the corpus callosum, as well as the cerebellar peduncles [185]. More recently, cortical demyelination has been detected [186], and demyelination in the hippocampal formation has been associated with seizures in the cuprizone model [187, 188]. Substantial demyelination is present starting after 3 weeks of cuprizone, and by 4–5 weeks more than 90% of axons will be demyelinated. Under continued exposure of cuprizone, about 50% of axons recover and are remyelinated by 6 weeks. If cuprizone is discontinued by this time, recovery continues, so that by 10 weeks 90% of the axons are remyelinated. If, in contrast, the cuprizone challenge is retained, demyelination again dominates so that few myelinated fibers remain [182]. During cuprizone toxicity, there is also demyelination-associated axonal loss which is more prominent in aged mice [189]. Reduction in average axon caliber and some dystrophic neurites are observed [190, 191]. Demyelination/remyelination is preferentially quantitated by ultrastructural analysis performed by electron microscopy, which is best to evaluate the number of myelinated axons, thickness of the myelin sheath, and relationship to the axon size. Histochemical lipid staining with Luxol Fast Blue–periodic Schiff or immunostaining for myelin proteins allows us to estimate and screen for myelination semiquantitatively. Biochemical (lipid) assays analyze the content of the myelin sheath [192]. Magnetic resonance imaging (MRI) has been used to map the pattern of demyelination noninvasively [193]. During administration of cuprizone, the expression of myelin genes in the brain decreases, and most of the mature oligodendrocytes in the corpus callosum, the site of particular profound demyelination, undergo apoptosis [194]. Studies of changes in oligodendrocyte precursor and mature oligodendrocyte dynamics suggest that oligodendrocyte precursors drive the repopulation of oligodendrocytes in the remyelination phase [194]. Oligodendrocyte precursor cells are detectable in the corpus callosum, presumably proliferate, and convert to mature glutathione S-transferase (GST)-$\Pi^+$ oligodendrocytes during remyelination [194], sequentially expressing myelin proteins [195].

In contrast to EAE, the BBB seems to remain intact [196, 197]; T cells are almost completely absent, and microglia/macrophages accumulate and predominate in the demyelinated areas [198, 199]. Bone marrow studies indicate that resident microglia outnumbers peripheral macrophages that are recruited in cuprizone-induced CNS demyelination [197]. Studies using lymphocyte-deficient RAG-1 mice have demonstrated that lymphocytes may not play a role in the cuprizone model [200, 201]. Microglia/macrophages are the main cell type responsible for clearance of myelin debris and are able to secrete a wide variety of cytokines. Analysis of MHC-II-null mice showed reduced inflammation, delayed remyelination, and regeneration of oligodendrocytes, whereas removal of MHC I had little effect [201, 202]. Macrophage depletion by treatment with clodronate liposomes throughout the remyelination phase significantly decreases the extent of oligodendrocyte remyelination following lysolecithin-induced demyelination [203]. Various inflammatory products, including IFN-$\gamma$, IFN-$\beta$, leukemia inhibitory factor, lymphotoxin-$\alpha$, lymphotoxin-$\beta$, IL-1$\beta$, nitric oxide, insulin-like growth factor, and macrophage inflammatory protein 1$\alpha$, have been studied in the cuprizone model and have been shown to have a deleterious or protective role [204–214]. TNF-$\alpha$ seems to be protective, promoting proliferation, differentiation of oligodendrocyte progenitors (bromodeoxyuridine-labeled $NG2^+$ cells), and subsequent remyelination [200]. Thus, induction of MHC II by TNF-$\alpha$ has been suggested as an important regulatory event in remyelination, emphasizing the active inflammatory response in brain regeneration after brain injury [202].

Neuropathological changes observed during cuprizone treatment correlate with alterations in the neurological function such as hyperactive behavior and mild motor dysfunction detectable by sensitive motor tests [215, 216].

## Model of lipopolysaccharide-induced demyelination

A number of studies have examined the effects of lipopolysaccharide (LPS) injections into the CNS [217–220]. Typically, a local inflammatory reaction is evoked but substantially muted when compared with similar injections in other tissues [217]. Felts et al. described an inflammatory reaction and after a delay of 5–7 days a demyelinating lesion that arise following the injection of LPS into rat spinal white matter [219, 220] and that persist between 9 and 14 days. Besides activation of resident microglia, CNS-infiltrating phagocytes (neutrophils and monocytes) seem to contribute to the inflammatory response [221], although injecting LPS or leukocyte chemotaxis into the CNS seems to induce a lower myelomonocytic recruitment compared to other tissues [217, 222]. Microglial TLR4 is required for leukocyte recruitment into the brain in response to LPS [223]. The exact mechanism of LPS-induced demyelination is unknown. In primary mixed glial cultures, LPS induces a selective loss of oligodendrocyte precursors, and activation of microglia, but not astrocytes, is required for LPS toxicity [224, 225]. Conditioned media from LPS-exposed microglia (or astrocytes) injures oligodendrocyte progenitors [226]. TLR4 is necessary for LPS-induced oligodendrocyte injury in the CNS, although mRNA for the LPS receptors TLR4/CD14 is either absent or only found at very low levels

in oligodendrocyte precursors [224]. Thus, LPS might not be directly toxic to oligodendrocytes but cause demyelination via factors derived from activated microglia/macrophages (or astrocytes). Accordingly, recent evidence suggests that microglial peroxynitrite (the reaction product of nitric oxide and superoxide anion), or in the presence of astrocytes activation of TNF/TNFR signaling, play a key role in LPS-induced damage of developing oligodendrocyte in vitro [225, 227].

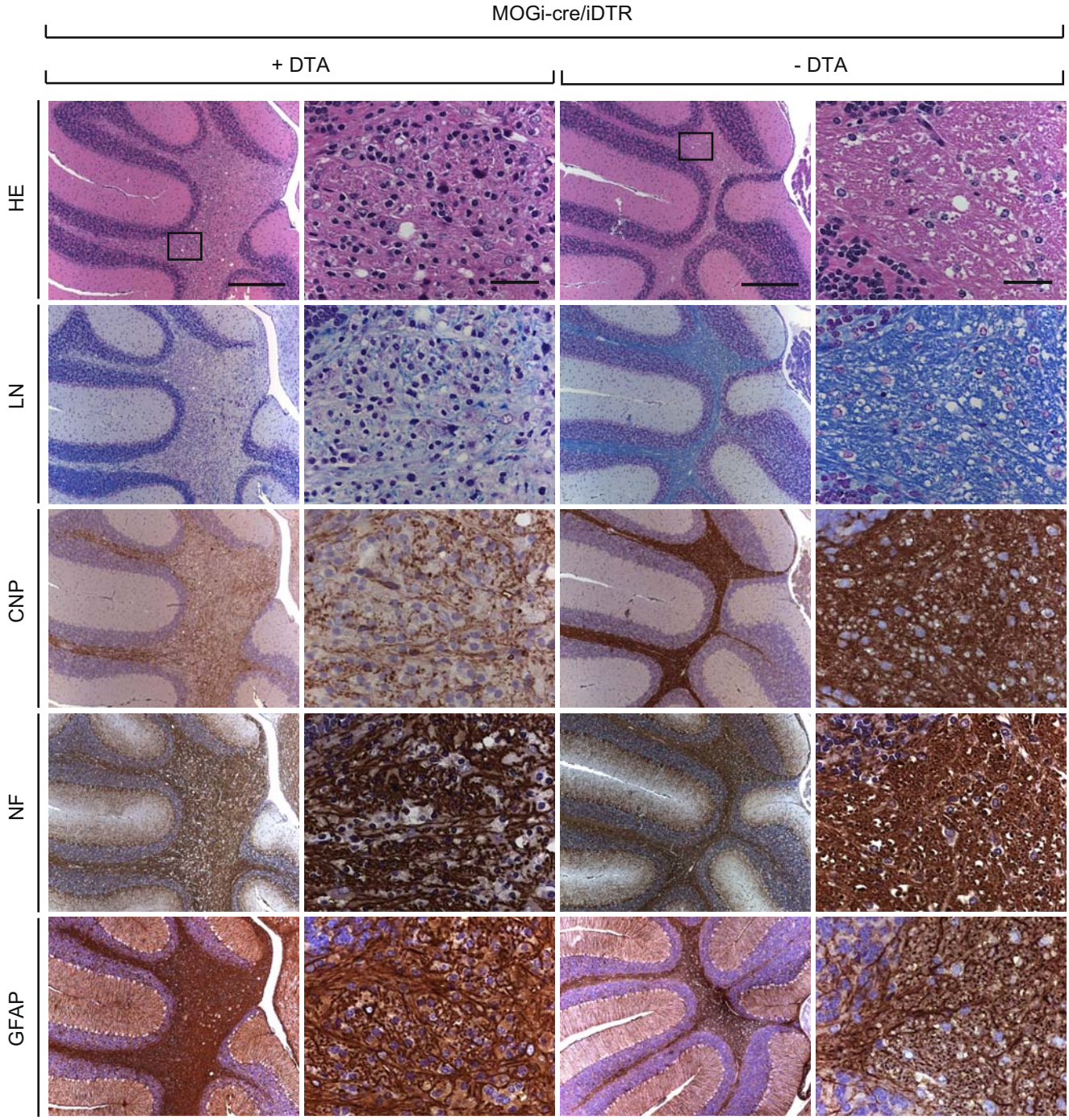


**Fig. 3** DT-mediated ablation of oligodendrocytes in MOGi-Cre/iDTR mice: cerebella of DT-treated (+DT, *first* and *second column*) MOGi-Cre/iDTR mice present with a severe destruction of myelinated structures, as visualized by Luxol-Nissl (*LN*) and CNPase stains. Untreated (−DT) iMOG-Cre/iDTR mice (*second* and *third column*) were unaffected. Axonal integrity was affected in DT-treated iMOG-Cre/iDTR mice (neurofilament immunohistochemistry) and some inflammatory cells (HE) and a mild reactive astrogliosis (GFAP) were visible in DT-treated iMOG-Cre/iDTR mice. *Rectangles* indicate the adjacent high-magnification image. *Scale bar* 500 μm for *first* and *third column*; 100 μm for *second* and *fourth column*. Adapted from [77]

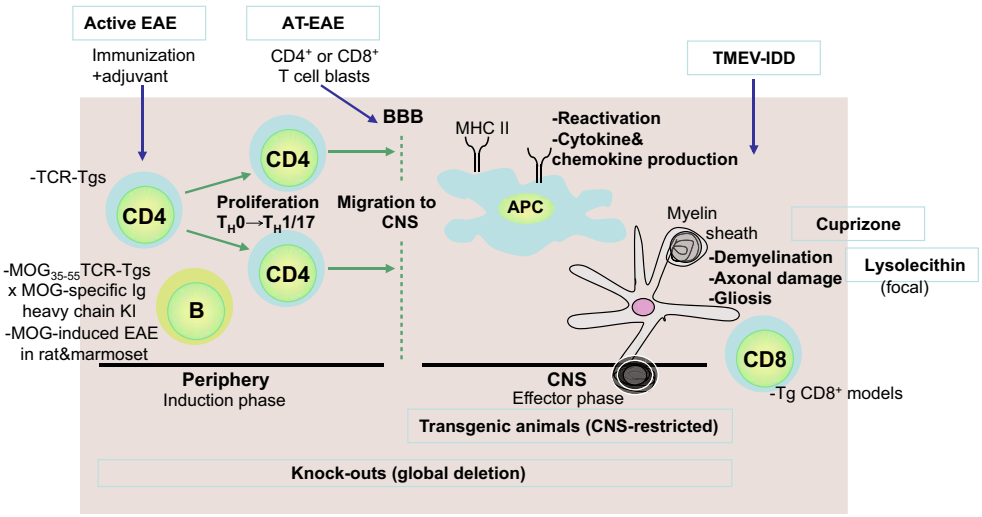


**Fig. 4** Induction and effector stage of MS/murine models. Abbreviations: *EAE*, experimental autoimmune encephalomyelitis; *AT*, adoptive transfer; *TMEV-IDD*, Theiler's murine encephalomyelitis virus-induced demyelinating disease; *TCR*, T cell receptor; *Tg*, transgenic; *KI*, knock-in, *KO*, knockout; $T_H$, T helper cell; *BBB*, blood–brain barrier; *MOG*, myelin oligodendrocyte glycoprotein; *MHC*, major histocompatibility complex

oDTR mice

The means for specific in vivo cell lineage ablation have recently been improved by stable mouse lines, carrying conditional expression constructs for DT or DTR that could be activated upon Cre-mediated recombination and the application of DT, respectively [77, 228]. In Cre-inducible DTR transgenic mice (iDTR) Cre-mediated excision of a STOP cassette renders cells sensitive to DT. DT crosses the BBB and promotes cell ablation in the CNS. Injecting DT intraperitoneally into MOGi-Cre/iDTR double transgenic mice expressing Cre recombinase under the control of the oligodendrocyte-specific promoter MOG results in a severe myelin loss throughout the CNS [77]. Axonal integrity subsequently also appears to be affected, and some reactive astrogliosis as well as inflammatory cells are seen (Fig. 3). After approximately 30 days, the DT-treated MOGi-Cre/iDTR mice present clinically with tremor, hind limb paralysis, and weight loss (Fig. 4; Table 1).

## Concluding remarks

Taken together, there is now a plethora of different animal models available, each of them serving the purpose to study specific aspects of the pathogenesis of MS in rodents. Whereas these models are most useful and have provided a wealth of knowledge otherwise unavailable to us, there are a number of limitations in regards to interpreting the data. A number of questions cannot be directly answered in animal models including the initial trigger of the MS pathogenesis (vaccination with autoantigen is hardly to blame), the driving self-Ag, the genetic predisposition, and others. Therefore, none of the described animal models can truly serve for drug testing or replace clinical trials. To this day, the animal models described here are the state-of-the-art and the very best modeling system available to us. We need to be cautious not to overinterpret the findings and to continue to create better models to accurately study certain aspects of the pathogenesis of neuroinflammatory disease.

**Table 1** Commonly used MS models

| Model | Mode of induction | Pathomechanism | Phenotype |
|---|---|---|---|
| EAE | -Immunization | $CD4^+$ T-cell-mediated | Myelitis (>cerebellitis) |
| | -Adoptive transfer ($CD4^+$) | ” | ” |
| | -Spontaneous → (humanized) TCR Tgs (MHCII-restricted) | ” | |
| | •2D2 $MOG_{35-55}$ TCR Tgs × MOG-specific IgH KI | ” | Optic neuritis [33] |
| | | $CD4^+$ T/B-cell-mediated | Devic-like disease [34] |
| | -In rats/marmosets (MOG) [in (conditional) Tg/KO mice] | T cell/antibody-mediated | Encephalomyelitis |
| $CD8^+$ AT | -Adoptive transfer ($CD8^+$) | $CD8^+$ T-cell-mediated | Brain > spinal cord [21] |
| | •TCR Tgs (MHCI-restricted) × auto/neo brain ag [spontaneous] | ” | Encephalomyelitis |
| TMEV-IDD | -Intracerebral infection | Virus-induced demyelination | Encephalomyelitis |
| Cuprizone | -Feeding | Toxin-based demyelination | Corpus callosum, cerebellar peduncles [185] |
| Lysolecithin | -Local CNS injection | ” | Focal white matter lesion |

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